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Time and Concentration Dependence of the Dicyclohexylcarbodiimide Inhibition of Proton Movements in the Cytochrome bc_1 Complex from Yeast Mitochondria Reconstituted into Proteoliposomes

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Abstract

The cytochrome bc_1 complex was isolated from yeast mitochondria solubilized with the detergent dodecyl maltoside and reconstituted into proteoliposomes to measure electrogenic proton pumping. Optimal respiratory control ratios of 4.0, obtained after addition of the uncoupler CCCP, and H^+/e^- ratios of 1.6 were obtained when the proteoliposomes were prepared with egg yolk phosphatidylcholine supplemented with cardiolipin. Moreover, it was critical to remove excess dodecyl maltoside in the final concentrated preparation prior to reconstitution to prevent loss of enzymatic activity. The rate of electrogenic proton pumping, the respiratory control ratios, and the H^+/e^- ratios were decreased by incubation of the cytochrome bc_1 complex with dicyclohexylcarbodiimide (DCCD) in a time and concentration dependent manner. Maximum inhibitions were observed when 50 nmol DCCD per nmol of cytochrome *b* were incubated for 30 min at 12°C with the intact cytochrome bc_1 complex. Under these same conditions maximum labeling of cytochrome *b* with [^{14}C] DCCD was reported in a previous study [Beattie *et al.* (1984). *J. Biol. Chem.* **259**, 10562–10532] consistent with a role for cytochrome *b* in electrogenic proton movements.

Key Words: Mitochondria; cytochrome; dicyclohexylcarbodiimide; energy transduction.

Introduction

The cytochrome b–c_1 complex of the mitochondrial respiratory chain catalyzes electron transport from ubiquinol to cytochrome *c* coupled to

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electrogenic proton translocation. The electrochemical protein gradient generated across the membrane during this process can be used for the synthesis of ATP by the F_1-F_0-ATP synthetase localized in the inner mitochondrial membrane as well as for ion transport. Recently, we reported that dicylohexylcarbodiimide (DCCD),[3] the well-known carboxyl-modifying reagent, inhibited the proton-translocating device in the cytochrome b–c_1 complex from yeast mitochondria reconstituted into liposomes (Beattie and Villalobo, 1982). Subsequent studies indicated that the concentrations of DCCD which block electrogenic proton movements in the b–c_1 complex reconstituted into proteoliposomes had a minimal effect on ubiquinol: cytochrome *c* reductase activity of either the complex (Clejan and Beattie, 1983) or rat liver mitochondria (Clejan *et al.*, 1984). The lack of inhibition by DCCD of electron transfer activity coupled with the observation that DCCD does not increase the conductance of the membrane (Beattie and Villalobo, 1982) suggest that the primary effect of DCCD is on the proton translocation mechanism at this site of the respiratory chain. Subsequently, it was shown in this laboratory that radioactive DCCD binds selectively to cytochrome *b* in the b–c_1 complex isolated from yeast mitochondria, suggesting that this protein is involved in proton translocation at site 2 of the respiratory chain (Beattie and Clejan, 1982; Beattie *et al.*, 1984).

These previous studies involving the effects of DCCD on the cytochrome b–c_1 complex were performed with a complex isolated from yeast mitochondria by cholate extraction followed by ammonium sulfate fractionation (Siedow *et al.*, 1978; Sidhu and Beattie, 1982). A major problem with the cholate extraction method for the isolation of the b–c_1 complex is that large quantities of mitochondrial protein (50 g) are required to obtain reproducible purifications. Moreover, variable amounts of contamination by cytochrome *c* oxidase have been observed as well as occasional unexplained losses of activity. Alternative methods involving Triton X-100 have been developed to solubilize the cytochrome b–c_1 complex from beef heart or *Neurospora crassa* mitochondria (Engel *et al.*, 1980; Linke and Weiss, 1986); however, extraction with Triton X-100 was unsuccessful with yeast mitochondria as a complete loss of the iron–sulfur protein was observed with a consequent loss of enzymatic activity.

Recently, Ljungdahl *et al.* (1987) have developed a new purification of the cytochrome b–c_1 complex from yeast mitochondria as well as from other organisms. This method, which involves solubilization of the mitochondrial membrane with the detergent dodecyl maltoside (DM) followed by ion

[3] Abbreviations used: DCCD, *N,N'*-dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate; DM, dodecyl maltoside; bc_1 complex, ubiquinol: cytochrome *c* oxidoreductase; PMSF, phenylmethylsulfonyl fluoride; DBH_2, reduced form of 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone; DFP, diisofluorophosphate; CCCP, carboxyl cyanide *m*-chlorophenylhydrazene.

exchange chromatography, has been used successfully in our laboratory for the isolation of an enzymatically active cytochrome b–c_1 complex from moderate (5–6 g) amounts of yeast mitochondria. In the current study, we have developed a method to incorporate the complex isolated by this method into liposomes with the retention of the energy coupling mechanism, as indicated by respiratory control ratios of 4.0 or greater in the presence of the uncoupler CCCP and H^+/e^- ratios of 1.6. Both the rate of proton pumping and the respiratory control ratio were decreased by prior treatment of the b–c_1 complex with DCCD in a time and concentration dependent manner equivalent to that previously reported for the covalent labeling of cytochrome b with [^{14}C]DCCD (Beattie *et al.*, 1984). These results provide further evidence that cytochrome b plays a role in the movement of protons across the membrane.

Materials and Methods

Red Star yeast was obtained from Universal Foods, Vista Industrial Park in Pittsburgh, Pennsylvania. Cells were broken using a Dyno Mill, Type DK-L containing glass beads approximately 0.45–0.50 mm in diameter (B. Braun, California). The anion exchange gels, DEAE Biogel A and DEAE-Sepharose CL-6B, were obtained from Biorad and Pharmacia, respectively. DCCD, ultrapure, biological grade, was purchased from Schwarz-Mann; dodecyl maltoside was from Calibiochem; asolectin was from Fluka and all other phospholipids from Sigma.

Preparation of Mitochondria and Submitochondrial Particles

Mitochondria were prepared from Red Star yeast incubated overnight at 20° in a medium (KPE) containing 0.9% KCl, 50 mM K_2HPO_4, pH 7.6, and 1 mM EDTA prior to isolation of mitochondria as described previously (Clejan Beattie, 1986). The mitochondrial pellets were resuspended to a protein concentration of 14–15 mg/ml in KPE buffer to which 0.5 mM PMSF was added just prior to sonication. Sonication was performed on 50 ml samples at 4° with a Q Horn Flat tip in a rosette cup three times for 45 sec at 40% output. The sonicated mitochondria were centrifuged twice at 8,000 × g for 10 min, and then at 100,000 × g for 60 min to obtain submitochondrial particles. The submitochondrial particles were washed by resuspending the pellet with buffer containing 50 mM Tris-HCl, 1 mM $MgSO_4$, 1 mM PMSF, and 1 mM DFP, pH 8.0 to 20 mg/ml, readjusting the pH to 8.0, and then centrifuging the submitochondrial particles at 100,000 × g for 90 min. The resultant pellet was resuspended to 10 mg/ml in the same medium prior to solubilization with DM.

Optimal detergent/protein ratios required to solubilize the cytochrome bc_1 complex were determined by addition of various amounts of DM (0.3, 0.4, 0.6, and 0.8 mG DM/mg protein) to the washed submitocondrial particles followed by centrifugation at 100,000 × *g* for 60 min. The total cytochrome *c* reductase activity in both the supernatant and the pellet was then determined.

Purification of the Cytochrome bc_1 Complex

Purification of the bc_1 complex was performed according to the method of Ljungdahl *et al.* (1986). This method involves DEAE BioGel A followed by DEAE Sepharose CL-6B ion exchange chromatography. The fractions eluted from the second column were analyzed spectrally and those with a maximum content of cytochromes *b* and c_1 and a minimum content of cytochrome a–a_3 were pooled. The pooled fractions were concentrated to a volume of approximately 1.0 ml using either an Amicon Diaflo PM 30 or XM300 membrane, then diluted 1 : 1 with glycerol and stored at −70°C.

Ubiquinol-cytochrome *c* oxidoreductase activity was assayed with the reduced decyl analog (DBH_2) of coenzyme Q (20 μM) as substrate in a medium containing 250 mM sucrose, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4, 2 mM KCN, and 20 μM cytochrome *c* using the wavelength pair 550/539 to determine the rate of cytochrome *c* reduction. The reaction was allowed to proceed for 20 sec and then 2 μM antimycin was added. Protein determinations were done by the method of Lowry (1951). The subunit composition of the isolated bc_1 complex was determined using SDS-polyacrylamide gel electrophoresis by the method of Laemmli (1970).

Production of Proteoliposomes

Egg yolk phospholipids (160 mg) containing 10% cardiolipin were dissolved in methanol and evaporated to dryness under nitrogen. The tube was placed in a vacuum desiccator and allowed to stand overnight. An 8-ml portion of a 3-mM K Hepes solution, pH 7.2, containing 100 mM KCl and 80 mg Na cholate was added to the phospholipids. Liposomes were made by sonicating the solution to clarify, 4 times using 30-sec pulses. A 1-mg portion of the bc_1 complex (containing approximately 3.6 nmol of cytochrome *b*) was added and the mixture sonicated once for 30 sec. The sample was then dialyzed for 18 h against 1 liter of 3 mM K-Hepes, pH 7.2, containing 100 mM KCl, with two buffer changes.

Proton ejection was measured in a medium containing 100 mM KCl, 0.1 mM EDTA, and 2 mM Hepes, pH 6.9, using a highly sensitive, small-diameter combination Ingold pH glass electrode in a 3-ml thermostated chamber at 20°C equipped with magnetic stirring. The reaction mixture contained 150 μg of the cytochrome b–c_1 complex, 2 μg valinomycin, 1 mM of

the reduced decyl analog of ubiquinol (DBH_2) as electron donor, and catalytic amounts of cytochrome *c* (20 μg). The reaction was initiated by addition of 20 or 40 nmol of $K_3Fe(CN)_6$. The instrument was calibrated by adding 10 and 20 nmol of HCl.

The respiratory control ratio was calculated by determining the rate of cytochrome *c* reduction by proteoliposomes (containing 30–40 μg of b–c_1 complex protein) in the medium described above in the presence of the uncoupler CCCP and dividing by the rate obtained in the absence of CCCP.

Effects of DCCD on the Isolated Cytochrome bc_1 Complex

The concentration dependence of DCCD to inhibit proton pumping was determined by incubating 500 μg of the bc_1 complex in a medium containing 100 mM KCl and 5 mM Hepes, pH 7.2, at 12°C with shaking with various amounts of a 4 mM stock DCCD solution in methanol to yield 25, 50, and 100 nmol DCCD/nmol cytochrome *b*. The reaction was stopped by the addition of 4 ml of the egg yolk phospholipid solution containing cardiolipin (40 mg/ml) to prepare proteoliposomes as described above. The extent and rate of proton pumping as well as the respiratory control ratio were determined. The time course of DCCD inhibition of proton pumping was measured by incubating 500 μg of the bc_1 complex with 50 nmol DCCD/nmol cytochrome *b* for 15, 30, and 60 min at 12°C with shaking. The egg yolk phospholipid plus cardiolipin mixture was added directly to stop the reaction by effectively diluting the DCCD. Proteoliposomes were prepared followed by dialysis. Both the extent and rate of proton pumping as well as the respiratory control ratio were determined.

Results

Before attempting to purify the cytochrome b–c_1 complex by the method of Ljungdahl *et al.* (1987), it was necessary to determine the amount of detergent required for optimal solubilization of the complex. Mitochondria obtained from breakage of yeast cells with glass beads in the Braun homogenizer were initially converted to submitochondrial particles prior to solubilization, thus removing a significant amount of the protein initially present. Addition of 0.3 mg of DM per milligram of submitochondrial protein resulted in solubilization of approximately half of the activity in the starting particles into a high-speed supernatant fraction (Fig. 1). Increasing the amount of DM to 0.6 mg/mg of protein resulted in solubilization of over 90% of the total cytochrome *c* reductase activity with a doubling of the total activity, while

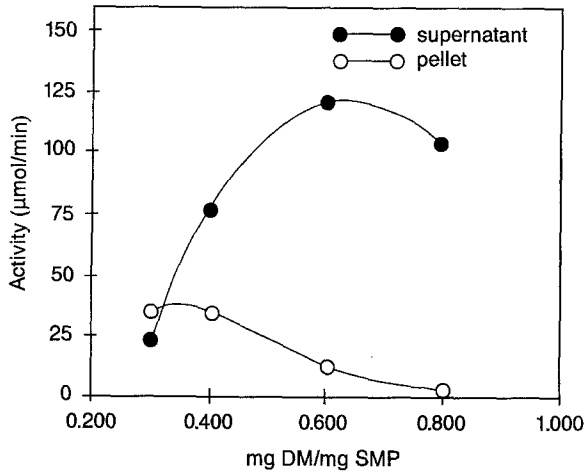

Fig. 1. Extraction of the bc_1 complex from yeast mitochondria with dodecyl maltoside (DM). Varying amounts of a DM solution (100 mg/ml) were added per milligram of submitochondrial particles suspended to concentration of 10 mg/ml for 30 min at 0°C followed by centrifugation at 100,000 × *g* for 60 min. DBH_2 cytochrome *c* reductase activity was determined as described under Materials and Methods in both the supernatant (●) and pellet (○) fractions.

higher amounts of DM resulted in a slight loss of activity. Consequently, 0.6 mg/mg of submitochondrial protein was used for solubilization prior to purification. The increase in total activity after addition of 0.6 mg of DM per mg of submitochondrial particles confirms previous reports that DM activates cytochrome *c* reductase activity (Ljungdahl *et al.*, 1987).

Table I presents a summary of the purification of the cytochrome bc_1 complex by this method, which involves two ion-exchange columns followed

Table I. Isolation of Yeast Cytochrome bc_1 Complex[a]

Fraction	Protein (mg)	Total activity (μmol/min/mg)	Specific activity (μmol/min/mg)	Fold purification
Submitochondrial particles	7000.0	1169	0.167	1.0
DM extract	1280.0	684	0.534	3.19
Pooled Biogel A column elutate	122.0	85	0.703	4.21
Pooled DEAE column eluate	45.5	217	4.78	28.6
Concentrated bc_1 complex	11.2	153	9.6	57.5

[a]The cytochrome bc_1 complex was isolated from submitochondrial particles obtained from yeast mitochondria as described by Ljungdahl *et al.* (1987). The enzymatic activity was determined as DBH_2: cytochrome *c* reductase activity.

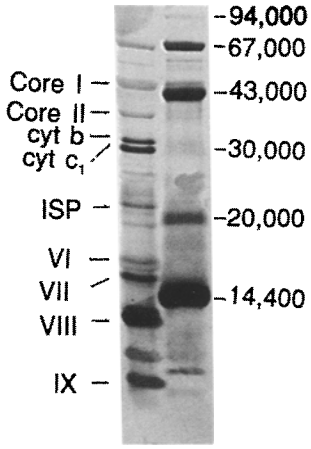

Fig. 2. SDS polyacrylamide gel of cytochrome bc_1 complex isolated from yeast mitochondria. The purified bc_1 complex was suspended in dissociating buffer and incubated overnight at room temperature prior to electrophoresis in a 15% polyacrylamide gel. The molecular weights of the different subunits of the bc_1 complex, indicated on the left, are 45,000, 40,000, 31,000, 29,000, 17,000, 14,000, 11,000, and 7,200.

by concentration through an Amicon Diaflo PM 30 membrane. A 57.5-fold increase in specific activity was observed in the purified complex compared to the activity in the submitochondrial particles. The specific activity was increased by another 25% after addition of 30 μg of DM per milligram of isolated complex. Interestingly, the detergent stimulation of enzymatic activity appeared to be limited to DM, as other detergents tested such as octyl-glucoside, cholate, or sodium dodecyl sulfate had no stimulatory effect at these concentrations (data not shown). The purified bc_1 complex contained nine clearly distinguishable subunits after electrophoresis on a 15% polyacrylamide gel (Fig. 2) with the apparent molecular weights indicated in the legend. Contaminants observed in variable amounts in this preparation include succinate dehydrogenase migrating at 67,000 Da and proteins at approximately 19,000 and 10,000 Da. The more sensitive silver staining method used on this gel indicates more apparent contaminants.

The bc_1 complex obtained by this procedure contained 2.1 μmol cytochrome *b* and 1.6 nmol of cytochrome c_1 per milligram protein, resulting in an apparent cytochrome *b* and c_1 ratio of 1.3 which is less than that expected for eukaryotic cytochrome bc_1 complexes (Ljungdahl *et al.*, 1986). The complex, however, contained negligible amounts of cytochrome a-a_3, was enzymatically active, and appeared adequate for further investigation of electrogenic proton movements.

Reconstitution of the bc_1 Complex into Liposomes

To study the proton pumping ability of the cytochrome bc_1 complex isolated after extraction of mitochondria with DM, the complex was reconstituted into liposomes by the method of Carroll and Racker (1977). This procedure involving sonication of a phospholipid/cholate solution to form phospholipid liposomes and subsequent addition of the complex followed by extensive dialysis to remove the detergent was used successfully in our previous studies with the bc_1 complex prepared by the cholate extraction method (Beattie and Villalobo, 1982; Clejan and Beattie, 1983). Our initial attempts to reconstitute the bc_1 complex isolated in the presence of DM led to the formation of proteoliposomes without detectable cytochrome *c* reductase activity. It is clear that DM present in the preparation does not inactivate the electron transfer activity of the complex as addition of DM to the isolated complex resulted in a stimulation of enzymatic activity; however, the presence of excess DM in the preparation might impede the formation of active proteoliposomes. The complex as isolated is concentrated by filtration through an Amicon PM30 membrane which excludes DM and thus would result in a 10-fold increase in the DM concentration to approximately 1 mg/ml from the 0.1 mg/ml concentration in the elution buffer. The excess DM was effectively removed by using an XM-300 filter. After concentration with this filter, the bc_1 complex retained antimycin-sensitive enzymatic activity and could be reconstituted into proteoliposomes prepared with the specific phospholipid as described below.

The effect of different phospholipids on the enzymatic activity of the bc_1 complex was investigated by incubating the complex for 1 h with liposomes prepared from various phospholipids prior to assaying for enzymatic activity. This procedure permitted the bc_1 complex to interact with the phospholipids and avoid the overnight dialysis step necessary to form proteoliposomes. The phospholipids used in these studies all contained various amounts of phosphatidylcholine as well as phosphatidylethanolamine and some minor phospholipids. For example, soybean type II-S contains 10–20%, soybean type IV-S contains 40%, while egg yolk type IX-E contains 60% phosphatidylcholine. Figure 2 indicates that addition of the bc_1 complex to soybean phosphatidylcholine type II-S or asolectin liposomes resulted in a complete loss of cytochrome *c* reductase activity, while 30 to 50% of enzymatic activity was retained after addition of the complex to the other phospholipids. Maximum enzymatic activity was observed in the presence of egg yolk phosphatidylcholine which was therefore used for the preparation of liposomes in all subsequent studies.

The cytochrome bc_1 complex reconstituted into these liposomes was capable of proton translocation with H^+/e^- ratios of 1.1–1.2. Previous

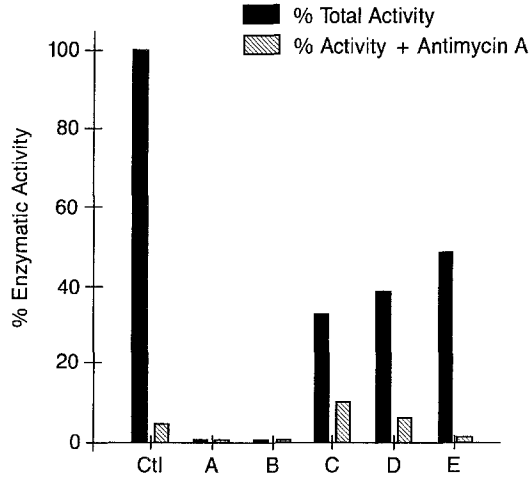

Fig. 3. Effects of phospholipid on the activity of the bc_1 complex. Different phospholipids were tested for their effects on the specific activity and antimycin A sensitivity of the cytochrome bc_1 complex. Ctl represents control; A, soybean phosphatidylcholine, type II-S; B, asolecithin; C, soybean phosphatidylcholine : phosphatidylethanolamine (9 : 1); D, soybean phosphatidylcholine, type IVS; E, egg yolk phosphatidylcholine, type IX-E.

studies (Fry and Green, 1981) had demonstrated that the acidic phospholipid, cardiolipin, is an absolute requirement for enzymatic activity of the bc_1 complex reconstituted into liposomes. The addition of various concentrations of cardiolipin to the egg yolk phosphatidylcholine prior to reconstitution of the bc_1 complex resulted in a stimulation of proton pumping activity without any effect on cytochrome *c* reductase activity (Fig. 3). The optimal pH for maximizing proton pumping activity was also determined to be 6.9 (data not shown). Sharp decreases in H^+/e^- ratios were observed at pH 6.7 or 7.4. The characteristics of the cytochrome bc_1 complex reconstituted into proteoliposomes with 10% cardiolipin added to egg yolk phosphatidylcholine and assayed at pH 6.9 are summarized in Table II. The optimal H^+/e^- ratios

Table II. Characteristics of Cytochrome bc_1 Complex Reconstituted into Liposomes[a]

Cytochrome *c* reductase	μmol/min/mg
Activity	2.3
+ Antimycin A	0.16
+ CCCP	9.2
H^+/e^- ratio	1.6

[a] The cytochrome *c* reductase activity and the H^+/e^- ratios were determined as described under Materials and Methods.

obtained using this procedure were 1.6. Moreover, the respiratory control ratio, defined as the stimulation of enzymatic activity after addition of the uncoupler CCCP, was 4.0, indicating that the bc_1 complex in the proteoliposomes is coupled. The retention of the energy coupling device in this b–c_1 complex preparation prompted us to reinvestigate the effects of DCCD on both proton pumping and electron transport.

Effects of DCCD on Proton Ejection in the Cytochrome bc_1 Complex Reconstituted into Proteoliposomes

Our initial investigations reporting the inhibitory effects of DCCD on electrogenic proton movements in the bc_1 complex from yeast mitochondria included a 1 h incubation of the complex with 250 nmol of DCCD/nmol cytochrome *b* (Beattie and Villalobo, 1982). Subsequently, we established that DCCD was bound selectively to cytochrome *b* in a time and concentration dependent manner (Beattie *et al.*, 1984). Maximum labeling of cytochrome *b* with radiolabeled DCCD was observed after a 30-min incubation of the bc_1 complex with 50 nmol DCCD/nmol of cytochrome *b*. Incubation of the complex with higher concentrations of DCCD resulted in no further incorporation of radioactive DCCD into cytochrome *b* and increased the nonspecific labeling of other subunits.

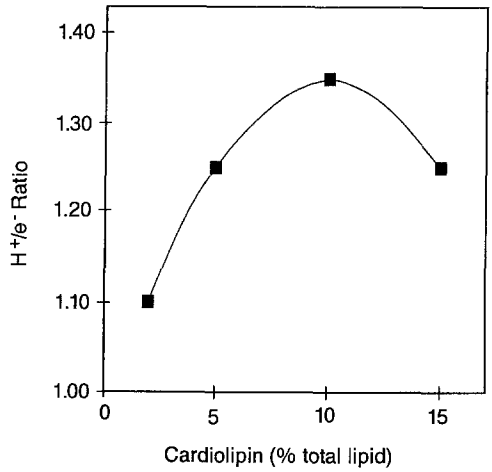

Fig. 4. Effects of cardiolipin on proton pumping activity in the cytochrome bc_1 complex. Proteoliposomes were made containing various amounts of cardiolipin: 2%, 5%, 10%, and 15%. The rate of proton pumping was calculated at 25°C in a 2.5 ml reaction mixture containing 150 μg of bc_1 complex protein, 1.07 mM DBH_2, 2 μg valinomycin, and 20 μg cytochrome *c*. The reaction was initiated by addition of 20 nmol $K_3Fe(CN)_6$.

We decided to resolve the discrepancies in these experiments by establishing the effects of both incubation time and DCCD concentration on respiratory control ratio and the rate of proton release in the b–c_1 complex reconstituted into liposomes (Fig. 4). Addition of 50 nmol of DCCD to the bc_1 complex resulted in a 35% decrease in the respiratory control ratio and an 80% decrease in the rate of proton pumping with minimal effects on cytochrome c reductase activity. The H^+/e^- ratio was decreased by 50% at this concentration, indicating a loss in electrogenic pumping activity. Increasing the concentration of DCCD to 100 nmol abolished respiratory control as indicated by the decrease in the respiratory control ratio of 1.0. In addition, the rate of proton pumping was inhibited by greater than 90%, a reflection of the 50% decrease in ubiquinol : cytochrome c reductase activity at this concentration of DCCD.

The time course of inhibition of both respiratory control and proton pumping was then investigated using the optimal concentration of DCCD (50 nmol/nmol of cytochrome b). Figure 5 indicates that a 30-min incubation of the bc_1 complex with this amount of DCCD was sufficient to decrease the respiratory control ratio of 1.0 and the H^+/e^- ratio by 50%; however, the ratio of proton pumping was decreased by 60% after a 15-min incubation with DCCD.

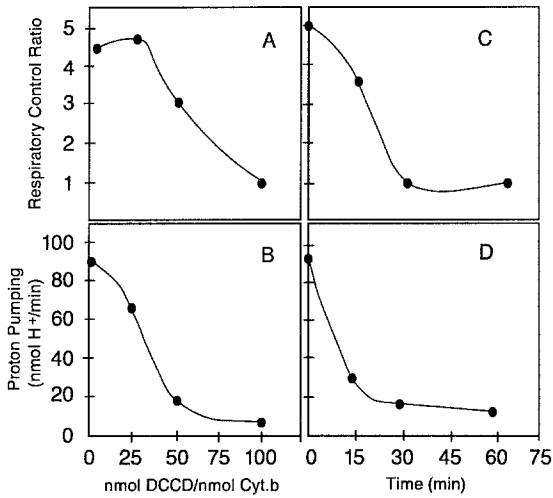

Fig. 5. Concentration dependence and time course of DCCD inhibition of electrogenic proton pumping in the cytochrome bc_1 complex reconstituted into liposomes. Varying amounts of DCCD (0, 25, 50, or 100 nmol/nmol cytochrome b) were incubated with the bc_1 complex for 1 h at 12° prior to production of liposomes (A and B). Alternatively, the bc_1 complex was incubated for various times with 50 nmol DCCD/nmol cytochrome b prior to production of liposomes (C and D). Respiratory control is cytochrome c reductase activity in the presence of CCCP divided by the activity in the absence of CCCP (A and C). The rate of proton pumping was measured using HCl to calibrate the pH electrode (B and D).

Discussion

During the past two decades, several methods have been developed for the isolation of the cytochrome b–c_1 complex (ubiquinol : cytochrome c oxidoreductase) from the mitochondria of various organisms involving the use of different detergents to solubilize the complex from the inner membrane. Unfortunately, the detergent Triton X-100 which has been widely used in the isolation of the b–c_1 complex from beef heart mitochondria (Engel *et al.*, 1980) cannot be used successfully to solubilize the b–c_1 complex from yeast mitochondria as the iron–sulfur protein is dissociated from the complex in the presence of this detergent. Recently, the non-ionic detergent, DM, has been introduced as an effective agent in the solubilization and acitvation of many membrane proteins including cytochrome c oxidase (Thompson and Ferguson-Miller, 1983), rhodopsin (Knudsen and Hubbell, 1978), the photosynthetic reaction center (Kendall-Tobias and Siebert, 1982), and ubiquinol : cytochrome c oxidoreductases from many organisms (Ljungdahl *et al.*, 1987). In the current study, we have isolated the b–c_1 complex from yeast mitochondria isolated by the method of Ljungdahl *et al.* (1987) and established the conditions necessary for reconstitution of the complex into proteoliposomes such that both respiratory control and electrogenic proton pumping are retained.

Several important parameters were determined to be essential for the successful reconstitution of the b–c_1 complex into proteoliposoes. It is critical that the concentration of DM in the preparation be significantly reduced prior to the formation of proteoliposomes. Clearly, DM by itself does not inactivate enzymatic activity in the complex but instead can significantly stimulate the activity in the isolated complex in addition to doubling of activity observed in submitochondrial particles. The high concentration of DM resulting from the final concentration step using an Amincon PM30 filter may have interfered with the formation of active proteoliposomes. Once the DM was removed by filtration through an Amincon XM300 filter, enzymatic activity was retained and active proteoliposomes were successfully prepared.

The choice of phospholipid used to form the initial liposome was also critical to maintain the optimal enzymatic activity of the b–c_1 complex. Addition of the complex to liposomes prepared with egg yolk phosphatidylcholine which contains 60% phosphatidylcholine and minimal amounts of contaminating lipids as determined by thin layer chromatography (data not shown) resulted in retention of the highest cytochrome c reductase activity. Further addition of cardiolipin to the phospholipid mixture prior to formation of liposomes resulted in the optimal $H^+/2e^-$

ratios provided that the pH of the reaction mixture was maintained at pH 6.9. Under these conditions, a respiratory control ratio determined as the rate of cytochrome *c* reductase in the presence of CCCP divided by that in the absence of CCCP (Leung and Hinkle, 1975) of 4.0 was obtained and a $H^+/2e^-$ ratio of 1.6. These values are similar to those that we reported earlier using a complex obtained by solubilization of yeast mitochondria with cholate (Beattie and Villalobo, 1982), although we were able to prepare active proteoliposomes with this preparation using either asolectin (Beattie and Villalobo, 1982) or type II-S phosphatidylcholine (Clejan and Beattie, 1983).

In the current study, we have confirmed the inhibitory effects of DCCD on proton translocation in the cytochrome b–c_1 complex using the complex isolated by the method of Ljungdahl *et al.* (1987). DCCD inhibits the rate of proton movements in this complex reconstituted into proteoliposomes, resulting in a decrease in the H^+/e^- ratio and in the respiratory control ratio with minimal effects on cytochrome *c* reductase activity at low concentrations of DCCD. Moreover, we have now established that the time course of DCCD inhibition of electrogenic proton movements as well as the concentration dependence for maximum inhibition correlates exactly with that previously reported for the binding of radiolabeled DCCD to cytochrome *b* (Beattie *et al.*, 1984). These results plus the observed 1 : 1 stoichiometry of binding of DCCD to the b–c_1 complex (Beattie and Clejan, 1982) are consistent with a role for cytochrome *b* in the translocation of protons across the mitochondrial membrane during electron transfer through this region of the electron transport chain.

DCCD, a carboxyl-modifying reagent, reacts with specific glutamate and aspartate residues in hydrophobic regions of membrane-spanning enzyme complexes and in so doing blocks proton translocation. The bulk of evidence of proton channels has been obtained with the ATP synthetase complexes of mitochondria, chloroplasts, and bacterial membranes where DCCD has been shown to bind to an acidic residue in the proteolipid subunit of F_0 (Fillingame, 1980). Evidence has also been obtained that DCCD blocks a proton channel associated with subunit III of cytochrome *c* oxidase without significant effect on electron transfer (Casey *et al.*, 1989; Prochaska *et al.*, 1981). More recently, Yagi (1987) has reported that NADH : ubiquinone reductase activity is also inhibited by DCCD, suggesting that similar mechanisms for the translocation of protons across the membrane exist at all three energy-transducing sites of the electron transport chain. Our current investigation on the topology of cytochrome *b* in the membrane and the site of DCCD binding may help in our understanding of the pathways of proton movements through the membrane.

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